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U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER

TRANSMITTAL LETTER TO THE UNITED STATES  
DESIGNATED/ELECTED OFFICE (DO/EO/US)  
CONCERNING A FILING UNDER 35 U.S.C. 371

1556.0290000/RWE

U.S. APPLICATION NO. (IF KNOWN, SEE 37 C.F.R. § 1.5)

(to be assigned) 09/869159

INTERNATIONAL APPLICATION NO

PCT/CA99/01235

INTERNATIONAL FILING DATE

23 December 1999

PRIORITY DATE CLAIMED

24 December 1998

TITLE OF INVENTION

ASSAY FOR IDENTIFYING COMPOUNDS WHICH AFFECT STABILITY OF mRNA

APPLICANT(S) FOR DO/EO/US

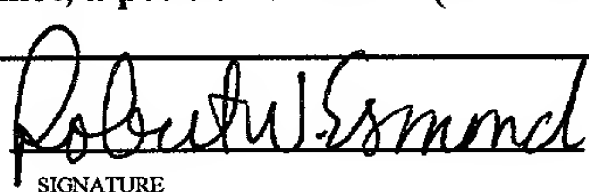
KASTELIC, Tania; CHENEVAL, Dominique

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)).
4. ☒ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
  - b. ☒ has been communicated by the International Bureau.
  - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International application under PCT Article 19 (35 U.S.C. 371(c)(3))
  - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
  - b. ☐ have been communicated by the International Bureau.
  - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
  - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 372(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern other document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.  
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
  - a. Copy of published International Application No. PCT/CA99/01235, including International Search Report;
  - b. Copy of International Preliminary Examination Report for International Application No. PCT/CA99/01235; and
  - c. Authorization To Treat A Reply As Incorporating An Extension Of Time Under 37 C.F.R. § 1.136(a)(3) (in duplicate).

U.S. APPLICATION NO. (if known, see 37 CFR 1.59) (to be assigned) <b>09/869159</b>		INTERNATIONAL APPLICATION NO. PCT/CA99/01235		ATTORNEY'S DOCKET NUMBER 1556.0290000/RWE	
17. <input checked="" type="checkbox"/> The following fees are submitted:				CALCULATIONS      PTO USE ONLY	
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... <b>\$1000.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... <b>\$860.00</b>  International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... <b>\$710.00</b>  International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... <b>\$690.00</b>  International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) ..... <b>\$ 100.00</b>					
<b>ENTER APPROPRIATE BASIC FEE AMOUNT</b> =					
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 860.00	
Total Claims				\$ 130.00	
Independent Claims					
Multiple dependent claim(s) (if applicable)					
<b>TOTAL OF ABOVE CALCULATIONS</b> =				\$ 1,278.00	
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$ 639.00	
<b>SUBTOTAL</b> =				\$ 639.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$ -0-	
<b>TOTAL NATIONAL FEE</b> =				\$ 639.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). <b>\$40.00</b> per property				\$ -0-	
<b>TOTAL FEES ENCLOSED</b> =				\$ 639.00	
				<b>Amount to be refunded:</b>	\$
				<b>charged:</b>	\$
a. <input checked="" type="checkbox"/> A check in the amount of <u>\$639.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. _____ in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>19-0036</u> . A duplicate copy of this sheet is enclosed. <b>NOTE: Where an appropriate time limit Under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>					
SEND ALL CORRESPONDENCE TO: <b>STERNE, KESSLER, GOLDSTEIN &amp; FOX P.L.L.C.</b> 1100 New York Avenue, NW, Suite 600 Washington, D.C. 20005-3934				 SIGNATURE  <u>Robert W. Esmond</u> NAME  <u>32,893</u> REGISTRATION NUMBER	
				<u>June 25, 2001</u> DATE	

09/869159

JCO3 Rec'd PCT/PTC 25 JUN 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Kastelic *et al.*

Appl. No. (U.S. National Phase of  
PCT/CA99/01235; U.S. Appl.  
No. (to be assigned))

Int'l Filing Date: December 23, 1999

For: **Assay for Identifying Compounds  
Which Affect Stability of mRNA**

Confirmation No.:

Art Unit: (To be assigned)

Examiner: (To be assigned)

Atty. Docket: 1556.0290000/RWE

**Preliminary Amendment**

Commissioner for Patents  
**Box PCT**  
Washington, D.C. 20231

Sir:

Prior to examination of the above-identified patent application, Applicants respectfully request entry of the following amendments. This Amendment is provided in the following format:

(A) A clean version of each replacement paragraph/section/claim along with clear instructions for entry;

(B) Starting on a separate page, appropriate remarks and arguments.

37 C.F.R. § 1.111 and MPEP 714; and

(C) Starting on a separate page, a marked-up version entitled: "Version with markings to show changes made."

It is not believed that extensions of time or fees for net addition of claims are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent

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abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

*Amendments*

*In the Specification:*

Please insert the following paragraph at page 1, after the title of the application:

Cross Reference to Related Application

This is the 371 national stage of International Application No. PCT/CA99/01235, filed December 23, 1999, published in English on July 6, 2000.

*In the Claims:*

Please substitute the following claims 7 and 10 for the pending claims 7 and 10:

7. (Once amended) An assay system comprising  
a stably transfected cell line according to claim 5, and  
a stably transfected cell line comprising a control DNA expression system  
which comprises a gene coding for expression of the protein having the detectable signal,

wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements but lacking any functional mRNA instability sequence.

10. (Once amended) A compound which destabilises mRNA identified by a method according to any one of claims 1-3.

Please add the following new claims 12-14:

12. (New) A compound which destabilises mRNA identified by use of a DNA expression system according to claim 4.

13. (New) A compound which destabilises mRNA identified by a cell line according to claim 5 or 8.

14. (New) A compounds which destabilises mRNA identified by an assay system according to claim 6, 7 or 9.

***In the Abstract:***

Please insert the Abstract enclosed on a separate sheet after the claims of the above-captioned application.

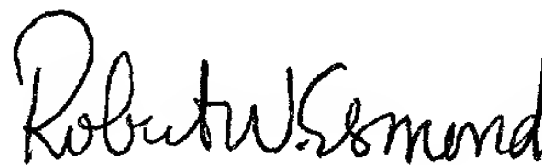
***Remarks***

The specification has been amended to add a section entitled "Cross Reference to Related Application" and an abstract on a separate sheet has been added. Claims 1-14 are pending in the above-captioned application, with claims 1, 3 and 4 being the independent claims. Claims 7 and 10 are sought to be amended herein to delete improper multiple dependencies; and new claims 12-14 are sought to be added. New claims 12-14 find support in original claim 10. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Applicants submit that this application is in condition for examination.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.



Robert W. Esmond  
Attorney for Applicants  
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Date: June 25, 2001

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09869159-081501  
T03T80-65T69860

**Version with markings to show changes made**

***In the Specification:***

A section entitled "Cross Reference to Related Application" was added at page 1 after the title of the application.

***In the Claims:***

Claims 7 and 10 were amended as follows:

7. (Once amended) An assay system comprising  
a stably transfected cell line according to claim 5, and  
a stably transfected cell line comprising a control DNA expression system  
[as defined in claim 6] which comprises a gene coding for expression of the protein  
having the detectable signal, wherein the gene comprises DNA coding for the amino acid  
sequence of the protein together with associated 5' and 3' UTR sequences comprising  
appropriate expression control elements but lacking any functional mRNA instability  
sequence.

10. (Once amended) A compound which destabilises mRNA [when]  
identified by a method according to any one of claims 1-3[, or by use of a DNA

expression system according to claim 4, a cell line according to claim 5 or 8, or an assay system according to claim 6, 7 or 9].

New claims 12-14 were added.

***In the Abstract:***

An Abstract on a separate sheet was added.

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JP03 Rec'd PCT/PTC 25 JUN 2001

WO 00/39314

PCT/CA99/01235

ASSAY FOR IDENTIFYING COMPOUNDS WHICH AFFECT STABILITY OF mRNA

This invention relates to an assay for the identification of biologically active compounds, in particular to a reporter gene assay for the identification of compounds which have an effect on mRNA stability.

Recently, it has become increasingly apparent that the regulation of RNA half-life plays a critical role in the tight control of gene expression and that mRNA degradation is a highly controlled process. RNA instability allows for rapid up- or down-regulation of mRNA transcript levels upon changes in transcription rates. A number of critical cellular factors, e.g. transcription factors such as c-myc, or gene products which are involved in the host immune response such as cytokines, are required to be present only transiently to perform their normal functions. Transient stabilisation of the mRNAs which code for these factors permits accumulation and translation of these messages to express the desired cellular factors when required; whereas, under non-stabilised, normal conditions the rapid turnover rates of these mRNAs effectively limit and "switch off" expression of the cellular factors. However, abnormal regulation of mRNA stabilisation can lead to unwanted build up of cellular factors leading to undesirable cell transformation, e.g. tumour formation, or inappropriate and tissue damaging inflammatory responses.

Although the mechanisms which control mRNA stability are far from understood, sequence regions have been identified in a number of mRNAs, which appear to confer instability on the mRNAs which contain them. These sequence regions are referred to herein as "mRNA instability sequences". For example, typical mRNA instability sequences are the AREs (AU rich elements), which are found in the 3'UTR (3' untranslated region) of certain genes including a number of immediate early genes and genes coding for inflammatory cytokines, e.g. IL-1 $\beta$  and TNF $\alpha$ .

As described in our copending British patent applications no. 9828707.1 and 9828710.5, we have discovered compounds which promote instability of mRNAs which contain mRNA instability sequences. Such compounds may be used to induce degradation of mRNAs, thus preventing or reversing inappropriate mRNA accumulation and thereby decreasing or preventing unwanted protein, e.g. cytokine, expression. Thus such compounds are potentially useful

pharmaceutically for prophylaxis or treatment of diseases or medical conditions which involve inappropriate mRNA stabilisation and accumulation and resultant undesirable protein expression.

The present invention relates to a reporter gene assay for identifying compounds which affect the stability of mRNAs which contain mRNA instability sequences.

Accordingly the present invention provides a method for the identification of a compound which affects mRNA stability, in which a DNA expression system which in the absence of the test compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, is contacted with a test compound and the detectable signal is measured in the presence of the test compound and compared with a control.

Preferably the method of the invention is adapted for the identification of compounds which promote instability of mRNAs which contain mRNA instability sequences. The reporter gene assay may be used to screen individual compounds and libraries of compounds, including combinatorial compound libraries. The reporter gene assay may be used as a first line screening assay to identify lead compounds and may be used to compare or quantify the mRNA instability promoting activity of compounds, e.g. to compare compounds produced from medicinal chemistry lead optimisation/ derivatisation programmes.

Thus in preferred embodiments the invention provides

- i) a method for the identification of a compound which induces mRNA degradation, comprising contacting the compound with a DNA expression system which in the absence of the compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of the test compound and comparing the result obtained with a control, or
- ii) a method for the comparison of compounds which induce mRNA degradation, comprising separately contacting the compounds with a DNA expression system which in the absence of the compounds is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of each test compound and comparing the signals obtained.

The DNA expression system typically comprises a gene coding for expression of the protein having a detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements including promoter and/or enhancer regions, and characteristically DNA corresponding to at least one copy of a mRNA instability sequence. Appropriate choice of promoter/enhancer sequences and other expression control sequences is a matter well within the ambit of the skilled worker in the art, and does not form a substantive part of the invention. Thus, for instance, for expression in mammalian cells a viral promoter such as an SV40, CMV or HSV-1 promoter may be used. On the other hand appropriate choice of mRNA instability sequence is of importance to the successful functioning of the reporter gene assay and forms part of the invention.

Thus in a further aspect the invention provides a reporter gene DNA expression system comprising a gene coding for expression of a protein having a detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements and DNA corresponding to at least one copy of a mRNA instability sequence.

mRNA instability sequences have been identified in the UTRs, in particular the 3' UTRs, of a large number of transiently expressed genes including genes for cytokines, chemokines, nuclear transcription factors, protooncogenes, immediate early genes, cell cycle controlling genes, oxygenases, genes involved in and controlling of apoptosis. The natural RNA sequences which comprise the mRNA instability sequences are alternatively referred to as adenylate/uridylate (AU)-rich elements, or AREs. Transiently expressed genes which contain mRNA instability sequences include, for example, the genes coding for GM-CSF, *c-fos*, *c-myc*, *c-jun*, *krox-20*, *nur-77*, *zif268*, *bcl-2*,  $\beta$ -IFN, uPA, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-13, TNF- $\alpha$ , MCP1, *syn1*,  $\beta_2$ -AR, E-selectin, VCAM-1, ICAM-1, Gro- $\alpha$ , Gro- $\beta$ , MMP-1, MMP-2, collagenases, P-glycoproteins (MDR), MRPs, *Pyh1* (pf mdr), COXII, and MIP-2 $\alpha$ .

The following publications include extensive discussion of mRNA instability sequences and AREs, the sequences motifs which they contain and (minimum) sequence requirements for

mRNA destabilisation, as well as identifying a number of mRNA instability sequences and the genes which contain them:

- Shaw & Kamen, Cell, Vol. 46, 659-667, August 29 1986 (GM-CSF);
- Shyu et al., Genes & Development, 5:221-231 (1991) (*c-fos*);
- Sachs, Cell, Vol. 74, 413-421, August 13 1993 (Review. "Messenger RNA Degradation in Eukaryotes");
- Chen et al., Mol. Cell. Biol., Jan 1994, p 416-426 (*c-fos*);
- Akashi et al., Blood, Vol. 83, No. 11, (June 1), 1994: pp 3182-3187 (GM-CSF etc.);
- Nanbu et al., Mol. Cell. Biol., July 1994, p. 4920-4920 (Upa);
- Stoecklin et al., J. Biol. Chem., Vol. 269, No. 46, November 18 1994, pp 28591-28597 (IL-3);
- Lagnado et al., Mol. Cell. Biol., Dec. 1994, p. 7984-7995 (general);
- Zhang et al., Mol. Cell. Biol., Apr. 1995, p. 2231-2244 (yeast);
- Zubiaga et al., Mol. Cell. Biol., Apr. 1995, p. 2219-2230 (general);
- Winstall et al., Mol. Cell. Biol., July 1995, p. 3796-3804 (*c-fos*, GM-CSF);
- Chen et al., Mol. Cell. Biol., Oct. 1995, p. 5777-5788 (*c-fos*, GM-CSF);
- Chen et al., TIBS 20 November 1995, 465-470 (review);
- Levy et al., J. Biol. Chem., Vol. 271, No. %, February 2 1996, pp. 2746-2753 (VEGF);
- Kastelic et al., Cytokine, Vol. 8, No. 10 (October), 1996: pp751-761;
- Crawford et al., J. Biol. Chem., Vol. 272, No. 34, August 22 1997, pp. 21120-21127 (TNF- $\alpha$ );
- Xu et al., Mol. Cell. Biol., Aug. 1997, Vol. 18, No. 8, p. 4611-4621 (general);
- Danner et al., J. Biol. Chem., Vol. 273, No. 6, February 6 1998, pp. 3223-3229 (human  $\beta_2$ -Adrenergic Receptor);
- Lewis et al., J. Biol. Chem., Vol. 273, No. 22, May 29 1998, pp. 13781-13786 (TNF- $\alpha$ );
- Chen, C.-Y. and Shyu, A.-B. Mol. Cell. Biol. Vol. 14, No. 12, 1994, pp. 8471-8482; and
- Klausner, R. et al., Cell, Vol. 72, 1993, pp. 19-28.

As described in the above publications mRNA instability sequences often contain one or more copies of sequence motifs, e.g. selected from:

AUUUA; UAUUUAU; UUAUUUA(U/A)(U/A), and AUUUAUUUA.

Thus mRNA instability sequence for use in the invention usually contains at least 1, preferably at least 2, or more preferably at least 3 of such sequence motifs or parts thereof (e.g. normally

containing at least 4 consecutive nucleotides from the motif) in appropriate juxtaposition, normally together, e.g. as tandem repeats, or with other, e.g. intervening, RNA sequences. Typically the mRNA instability sequence comprises from about 20 up to about 100 or more, preferably from about 30 to about 50, nucleotides in length. The mRNA instability sequence may be derived as a restriction fragment from the 3' UTR of an appropriate gene, or as a de novo synthesised nucleotide sequence. Alternatively the whole or a substantial part of the 3' UTR of an appropriate natural gene sequence, which contains a mRNA instability sequence may be used.

DNA corresponding to any mRNA instability sequences or AREs, including those described in the above publications, or functionally equivalent variants thereof, may be used in the DNA expression system of the invention. Preferably, however, the mRNA instability sequence used is one derived from the mRNA which codes for a protein which is implicated in the disease of interest. Thus, for example, a mRNA instability sequence for use in detecting compounds which destabilise the mRNA which codes for a cytokine or oncogene which is involved in the aetiology of a particular disease process, is preferably derived from the gene which codes for the cytokine or oncogene in question, e.g. lead compounds for treatment of IL-1 mediated diseases, such as rheumatoid arthritis or osteoarthritis are preferably detected using a reporter gene expression system comprising an IL-1 mRNA instability sequence.

Thus by way of illustration of the invention a preferred mRNA instability sequence for use in the identification of compounds which destabilise IL-1 $\beta$  mRNA is derived from the 3' UTR of IL-1 $\beta$  mRNA, e.g. the sequence shown in Figure 1. More preferably the IL-1 $\beta$  mRNA instability sequence may comprise a fragment of the 3' UTR of IL-1 $\beta$  mRNA. For example, a particularly preferred IL-1 $\beta$  mRNA instability sequence comprises the 30 nucleotide sequence derived from the 3' UTR of IL-1 $\beta$  mRNA (shown in Figure 2).

Preferably the mRNA instability sequence is located in the 3' UTR of the reporter gene. Thus for example, the DNA sequence corresponding to the mRNA instability sequence is inserted as or as part of an appropriate DNA segment into a suitable restriction site in the 3' UTR of the native reporter gene.

The DNA expression system is preferably a cell based expression system, conveniently in the form of a suitably transformed cell line, preferably a stably transformed cell line. The host

cell is typically an eucaryotic host cell, in particular an animal host cell, especially a mammalian host cell.

Preferably the host cell is of the same general cell type as the cells which express the protein which is coded for by the mRNA which it is desired to destabilise. Thus for instance, if the assay of the invention is to be used for the identification of compounds which destabilise the mRNA coding for a cytokine, the host cell used is preferably a cell or cell line which is of the same or similar cell type to the cells which normally produce the cytokine in question. For example, monocyte or monocyte-like cell lines may be used as host cells for assaying for compounds which destabilise cytokine, e.g. IL-1 $\beta$ , mRNA. Preferred cell lines for oncogene and other cancer related gene mRNA instability assays are, e.g. Colon 205, KB 31, KB 8511, DU-145, HCT116, MCF7, MCF7/ADR, MDA-MB-231, MDA-MB-435 and MDA-MB-435/TO. Particularly preferred cell lines for use as the host cells in assays of the invention for identification of compounds which destabilise cytokine, e.g. IL-1 $\beta$ , mRNA are the THP-1 cell line (for instance as described by Auwerx J. (1991), *Experientia*, 47: 22-30) and similar monocytic, e.g. human leukaemia, cell lines.

Preferably also, the mRNA instability sequence and the host cell are derived from the native mRNA which it is desired to destabilise and the native cell type in which that mRNA is produced respectively. Thus for instance, for identification of compounds which destabilise cytokine mRNA, the mRNA instability sequence is preferably derived from the mRNA which codes for the cytokine in question and the host cell is preferably of the same cell type as the native cell type in which the cytokine mRNA is produced. For example, for identification of compounds which destabilise IL-1 $\beta$  mRNA, the mRNA instability sequence is preferably derived from the 3' UTR of IL-1 $\beta$  mRNA and the host cells used are monocyte-type cells, e.g. THP-1 cells.

Although the mechanism of mRNA destabilisation, and the role of mRNA instability sequences in this, is not fully understood, it is clear that the presence of other factors besides the destabilising compound and the mRNA instability sequence are required for mRNA destabilisation to take place; for instance, as discussed in previously identified literature references. Conveniently such other factors are provided by the transformed host cell environment and complement or complete the interaction of the compound and the mRNA



instability sequence to effect destabilisation of the mRNA. Preferably the transformed host cells may be stimulated or otherwise activated to enhance mRNA destabilisation, e.g. to provided enhanced levels of the cellular factors required for mRNA destabilisation. In particular we have found that improved results are obtained in the assay of the invention if differentiated transformed host cells are used. For example, in the case of transformed THP-1 cells we have found that the best results are obtained if the transformed THP-1 cells are grown, differentiated and stimulated with  $\gamma$ IFN and LPS as is normal for THP-1 cells, e.g. as described hereinafter in the Examples.

The protein coded by the reporter gene mRNA may itself comprise the detectable signal. For instance, the protein may comprise a fluorescent protein, e.g. green fluorescent protein. Preferably, however, the protein is such that it is capable of reacting with an appropriate substrate or other substance to give a detectable signal. Conveniently the protein coded by the mRNA is an enzyme or enzymically active fragment of an enzyme. Examples of suitable enzymes include horseradish peroxidase (HRP), chloramphenicol acetyltransferase (CAT), alkaline phosphatase (AP), secreted alkaline phosphatase (SEAP),  $\beta$ -galactosidase, or especially luciferase. Methods for detecting and determining such enzymes are well-known, using appropriate substrates and measurements; for instance, as described hereinafter for the determining the levels of luciferase expression. It will be appreciated, however, that any suitable detectable protein and measurement procedure may be used.

In the assay of the invention the presence of a compound which destabilises mRNA is indicated by a decrease in the magnitude of the detectable signal given by the protein produced from the expression system in the presence of the compound as compared with a control; destabilisation of the reporter gene mRNA by the compound leads to a decrease in expression of the protein and thus a decrease in the magnitude of the signal. A suitable control for use in the assay of the invention comprises a DNA expression system which corresponds to the reporter gene DNA expression system, i.e. contains sequence coding for expression of the detectable protein but which does not contain sequence corresponding to a mRNA instability sequence. Preferably the control DNA expression system is identical to the reporter gene expression system except that the DNA corresponding to the mRNA instability sequence has been removed, deleted or otherwise disabled as a mRNA instability sequence. Preferably the control DNA expression

system is also in the form of a transformed cell line, typically a stably transformed cell line derived from the same host cell line, e.g. a THP-1 cell line, as the reporter gene transformed cell line.

Accordingly in a preferred embodiment the invention provides an assay system for the identification of compounds which destabilise mRNA comprising

a reporter gene DNA expression system as defined above, and

a control DNA expression system which comprises a gene coding for expression of the protein having the detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements but lacking a functional mRNA instability sequence.

Preferably both the reporter gene DNA expression system and the control DNA expression system are in the form of stably transfected cell lines.

Alternatively the reporter gene expression system may be tested in the presence and absence of the test compound, testing in the absence of the test compound being used as the control. In another alternative embodiment a control DNA expression system may also be present in the same cell line as the reporter gene DNA expression system. The control DNA expression system in this case would code for a detectable protein which is different than the protein coded for by the reporter gene expression system, and as before, the control DNA expression system lacks any functional mRNA instability sequence.

The invention is further described by way of illustration of the invention only in the following Examples which relate to a particular assay of the invention and refer to the accompanying Figures:

Figure 1 which shows the DNA sequence of IL-1 $\beta$  3' UTR;

Figure 2 which shows the 30 bp fragment used as a mRNA instability sequence in

Example 1;

Figure 3 which shows plasmid diagrams for pGL2\_Neo30 and pGL2-Control;

Figure 4 which shows graphs of luciferase activity over the time of differentiation for clone No. 53 (A) and clone No. 63 (B);



Figure 5 shows graphs of luciferase half lives, 4 and 8 hours after addition of compounds for clones 53 and 63 treated with radicicol analog A (SDZ 216-732), actinomycin D (act D.) and cyclohexamide (CHX);

Figure 6 shows graphs of luciferase activity from clones 53 (solid bars) and 63 (open bars) treated with various concentrations of radicicol analog A (SDZ 216-732);

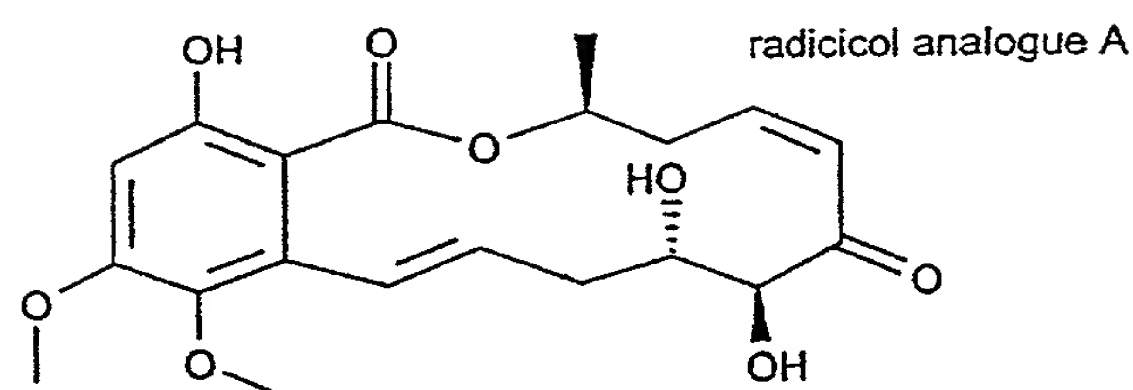
Figure 7 shows graphs of luciferase activity for undifferentiated (undiff) and differentiated (diff) clone 53 (solid bars) and clone 63 (open bars) treated with radicicol analog A, and

Figure 8 shows a graph of the concentration inhibition of luciferase activity by radicicol analog A.

### EXAMPLES

We have shown earlier (Kastelic et al., CYTOKINE. Vol. 8, No. 10 (October), 1996: pp751-761) that radicicol analog A (the compound shown below) confers mRNA instability through the AU-rich element (ARE) motifs located in the 3' untranslated region (3' UTR) of genes subject to mRNA instability. For these studies, the segment of 3' UTR of IL-1 $\beta$  which contains all the AREs was deleted and the resulting IL-1 $\beta$  -AU cDNA was subcloned into an expression vector. Stably transfected THP-1 cells containing this construct were analyzed by the RNase protection method (Kastelic et al. *ibid*) and showed resistance of the AU-less derived RNA towards radicicol analog A.

The 3'UTR of IL-1 $\beta$  mRNA contains a total of 6 AUUUA motifs three of which are in tandem (see Figure 1). For the construction of the luciferase reporter gene assay, we used only the a fragment comprising of the underlined sequence shown in Fig. 1 which contains three tandem repeats. Findings by Zubiaga et al (*ibid*) indicate that the minimal sequence of the mRNA instability motif is UUAUUUAUU (a sequence which occurs in the inserted 30 bp IL-1 $\beta$  fragment which we used) rather than just AUUUA alone.



### Example 1: Construction of pGL2\_Neo30 and stable cell lines

In order to obtain a vector for stable integration into THP-1 cells, a XhoI - SalI fragment of the neo resistant gene (expressing aminoglycoside 3' phosphotransferase) derived from pMCIneo (Stratagene) is subcloned into the SalI site of pGL2-Control (Promega). This resulting plasmid was called pGL2\_Neo. A 30bp fragment (containing three tandem AUUUA motifs, based on the IL-1 $\beta$  3'UTR sequence) obtained by annealing two complementary synthetic oligonucleotides (see Figure 2) is subcloned into pGL2\_Neo using the PflMI restriction site. This results in the luciferase expression vector pGL2\_Neo30 (Fig. 3). Fig. 2 shows the IL-1 $\beta$  3'UTR sequence containing three tandem AUUUA motifs used for ligation into the PflMI site of pGL2\_Neo. Expression vector pGL2- $\beta$ -galactosidase (Promega) has the *lacZ* gene driven by the same promoter (SV40) as the luciferase gene in pGL2\_Neo30 and pGL2\_Neo, but plasmid pGL2- $\beta$ -galactosidase does not contain any mRNA instability sequences.

THP-1 cells are then transfected with pGL2\_Neo vector (to generate control cell lines) or are cotransfected with pGL2\_Neo30 vector pGL2- $\beta$ -galactosidase by electroporation.  $10^7$  cells/ml in 1.3mM KH<sub>2</sub>PO<sub>4</sub>, 7.36mM Na<sub>2</sub>HPO<sub>4</sub>, 2.44mM KCl, 124mM NaCl, 5mM glucose, 9.6 $\mu$ M MgCl<sub>2</sub> and 16 $\mu$ M CaCl<sub>2</sub> pH 7.2 are transfected with 20 $\mu$ g of DNA in a Bio-Rad Gene Pulser (250V, 690 $\mu$ F and indefinite resistance) using a 0.4cm cuvette. Cells are subsequently cultured in RPMI medium containing 10%FBS, 2mM L-Gln (L-glutamine), 50 $\mu$ M 2-mercaptoethanol and 600 $\mu$ g/ml of G418 (geneticin). After transfection of pGL2\_Neo30 and pGL2\_Neo into THP-1 cells, stable cell lines are obtained by selection for G418 resistance and assayed for luciferase activity (and the cotransfected cell line is also assayed for  $\beta$ -galactosidase activity which can serve as an internal control – see Example 5 below). One cell line of each

transfection is chosen for further analysis; the pGL2\_Neo30/ pGL2- $\beta$ -galactosidase cell line is referred to as clone No. 63 and the pGL2\_Neo cell line as clone No. 53. No endogenous luciferase activity could be detected in normal THP-1 cells.

The tissue culture and luciferase activity measurements are carried out as described below.

#### Tissue culture:

The transfected human monocytic leukaemia cell lines, clones No. 53 and 63 are grown in RPMI medium supplemented with 110 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-Gln and 2 g/l NaHCO<sub>3</sub>. Heat-treated FBS (5%) is added before use. The cells are grown to a density of  $5 \times 10^5$ /ml and induced to differentiate with 100 U/ml (final concentration)  $\gamma$ IFN. Three hours later, 10  $\mu$ l of LPS (5 $\mu$ g/ml final concentration) is added. This time point is designated time 0. Compounds are added at various times after LPS addition as indicated.

#### Luciferase activity measurement:

In order to adapt the system to the use of 96 well plates, cells are grown in Packard flat bottom white polystyrene microplates (Cat. No.6005180) in RPM1 medium lacking phenol red (AMIMED). Cells are plated at  $5 \times 10^4$ /well. After treatment of the cells, luciferase is measured using the Packard Luc Lite system (Cat. No.601691 1) according to the manufacturer's instructions in a final volume of 205 $\mu$ l. Briefly, to a cell suspension of  $5 \times 10^5$  cells/ml,  $\gamma$ IFN (1000U/ml Boehringer Mannheim No. 1050494) to a final concentration of 100 U/ml and 0.25% (v/v) Luc Lite Enhancer is added. After a 3 hour incubation LPS (50 $\mu$ g/ml SIGMA L-8274) is added to give 5 $\mu$ g/ml final concentration. The cells are then plated at  $5 \times 10^4$ /100 $\mu$ l/well into flat bottom white polystyrene microplates (Packard, Cat. No. 6005180) and incubated for 16 hours. 5  $\mu$ l of compound solution or control vehicle is then added and the cells are further incubated as indicated. 100  $\mu$ l of luciferase substrate solution is added and the plates are covered with TopSeal-A press-on adhesive sealing film (Packard Cat.No. 6005185) before measuring

luminescence with a Packard Top Count Scintillation Counter at 22°C. The luciferase signal is stable for at least 90 min.

The differentiation-dependent induction of luciferase activity in the two cell lines, Nos. 53 (A) and 63 (B) are tested and the results obtained are shown in Figs. 4 A and B. In both clones a distinct induction of luciferase expression can be observed, maintaining high levels of activity throughout the time of the assay. This elevated and constant expression of luciferase should be born in mind when analyzing effects of compounds inducing mRNA instability. mRNA degradation will be in constant competition with de novo transcription, unlike the situation in wild-type THP-1 cells where in the case of IL-1 $\beta$ -mRNA, highest levels are obtained 16 hours after LPS addition. One would expect in the case of luciferase to see a weaker effect of mRNA destabilizing drugs since transcription remains high. Indeed this is what we observe in the case of radicicol analog A, see below.

#### Example 2: Half life of Luciferase mRNA and protein

To measure mRNA degradation using luciferase protein activity it is important to know the half life of the luciferase enzyme in order to determine an optimal time for assaying for potential mRNA destabilizing agents by way of luciferase protein stability. The possibility exists that mRNA could be degraded but due to a long half life of the protein, high enzyme activities could persist. Therefore we analyzed luciferase activities after addition of the transcriptional inhibitor actinomycin D (act. D) or the translational inhibitor cycloheximide (CHX). Fig. 5 shows that in the presence of 20 $\mu$ g/ml act.D as well as in the presence of 20  $\mu$ M CHX, luciferase activities rapidly decline and after 8 hours of incubation reach a level comparable to the inhibition achieved by radicicol analog A. In view of this relatively short half life of the luciferase enzyme, it is safe to assess any substance for activity on mRNA degradation as early as 8 hours after compound addition.

Example 3: Effect of the radicicol analog A

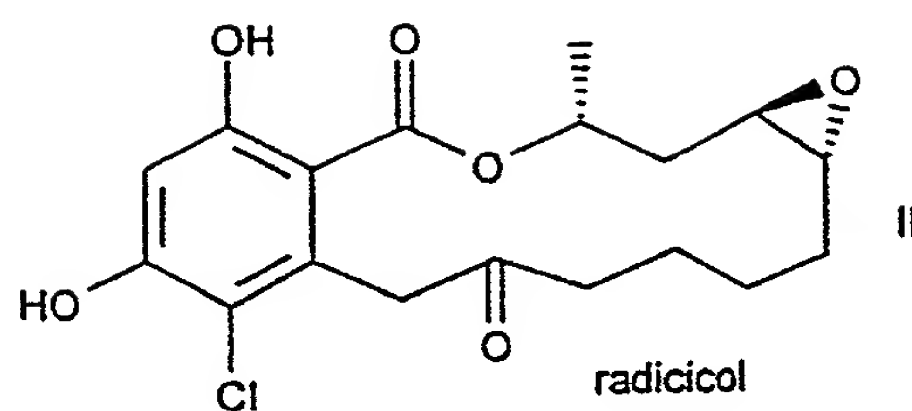
The THP-1 cell lines, clone No. 63 (containing pGL2\_Neo30) and clone No. 53 (containing pGL2-Neo) are grown, differentiated with  $\gamma$ IFN and stimulated with LPS identical to normal THP-1 cells. Radicicol analog A is added 16 hours after the addition of LPS and cell extracts are then taken 8 hours later or as indicated. Luciferase activity is inhibited by 1  $\mu$ M radicicol analog A on average by 50%  $\pm$  17%, in some cases inhibition was as great as 93%, whereas up to  $5 \times 10^{-6}$  M of radicicol analog A has no effects on the control clone No. 53, Fig. 6 (solid bars indicate clone No. 53, open bars clone No. 63).

Interestingly, undifferentiated (undiff) clone No. 63 (open bars) when treated with radicicol analog A showed only a limited reduction of luciferase activity (Fig. 7, solid bars indicate clone No. 53), which is either due to the lower expression of luciferase or is indicative of the involvement of a differentially expressed or modified component in the mRNA degradation process mediated by AU-rich elements. Indeed, gel retardation experiments using 241 bp of the AU-rich 3' UTR of IL-1 $\beta$  as a riboprobe showed the binding of additional proteins with  $\gamma$ IFN induced differentiation or modification (not shown).

Concentration dependent inhibition of luciferase activity is shown in Fig. 8. Concentrations of radicicol analog A higher than  $5 \times 10^{-6}$  M also inhibited the control clone due to cytotoxicity or inhibitory activity on transcription.

Example 4: Application of assay to a number of selected substances

A number of selected substances are tested for their activity in the assay of the invention substantially as described in Example 3 (for differentiated cells). The results obtained are given in the Table 1 below. Radicicol (see formula II below) and radicicol analog A show a clear effect on mRNA stability; other compounds tested did not show activity in the assay used.

**TABLE 1**

COMPOUND	Luciferase activity (% of control)	
	clone No. 53	clone No. 63
peptidic ICE inhibitor	87	104
stemphon	95	90
radicicol	98	47
(17 $\alpha$ )-23-(E)-dammara-20,23-dien-3 $\beta$ ,25-diol	116	91
radicicol analog A	120	49
thalidomide	98	112
dexamethasone	72	63
cyclosporin A	82	74

**Example 5: Application of assay using a single cell line**

In the previous examples, test compounds are assayed by comparing their activity in two separate cell lines (clone 53 and clone 63). However, clone 63 was cotransfected with two separated plasmids: one plasmid (pGL2\_Neo30) contains the luciferase gene with the 30 bp instability sequence driven by the SV40 promoter and the other plasmid (pGL2- $\beta$ -galactosidase) contains the *lacZ* gene driven by the SV40 promoter but contains no mRNA instability

sequences. The  $\beta$ -galactosidase activity of this cell line should not be effected by exposure of the cells to compounds which promote mRNA instability via mRNA instability sequences. As a result, one should be able (in theory) to screen for compounds having mRNA instability activity by simply comparing luciferase activity in unstimulated cells versus stimulated cells and comparing the  $\beta$ -galactosidase activity in these same cells. To test this hypothesis, the effect of radicicol analog A on luciferase activity and  $\beta$ -galactosidase activity in clone 63 (stimulated and unstimulated cells) was compared to the effect of radicicol analog A on stimulated and unstimulated cells of clone 63 and clone 53. The assay was performed as described in the previous Examples. Table 2 shows the luciferase activities of various concentrations of radicicol analog A in  $\gamma$ IFN/LPS stimulated and unstimulated cells of clones 63 and 53. Activities are given in % of control and are based on means of three independent experiments controlled for cell numbers. Table 3 shows the  $\beta$ -galactosidase activities in stimulated and unstimulated cells of clone 63. Activities are given in % of control and are based on means of three independent experiments controlled for cell numbers. It is clear from the data that both the assay of Table 2 and that of Table 3 would have identified radicicol analog A as an active compound.

**TABLE 2**

<b>Luciferase activity</b>				
	<b>clone 63</b>		<b>clone 53</b>	
	unstimulated	$\gamma$ IFN/LPS stimulated	unstimulated	$\gamma$ IFN/LPS stimulated
	(%control)	(%control)	(%control)	(%control)
No compound	100	100	100	100
1 $\mu$ M radicicol analog A	63	7	nd	88
10 $\mu$ M radicicol analog A	11	2	87	63

**TABLE 3**

<b><math>\beta</math>-galactosidase activity</b>				
	<b>clone 63</b>		<b>clone 53</b>	
	unstimulated	$\gamma$ IFN/LPS stimulated	unstimulated	$\gamma$ IFN/LPS stimulated
	(%control)	(%control)	(%control)	(%control)
No compound	100	100	100	100
1 $\mu$ M radicicol analog A	96	97	99	98
10 $\mu$ M radicicol analog A	84	70	103	62



CLAIMS

1. A method for the identification of a compound which affects mRNA stability, in which a DNA expression system which in the absence of the test compound is capable of expressing a protein having a detectable signal, and wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence is contacted with a test compound and the detectable signal is measured in the presence of the test compound and compared with a control.
2. A method according to claim 1, for the identification of a compound which induces mRNA degradation, comprising contacting the compound with a DNA expression system which in the absence of the compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of the test compound and comparing the result obtained with a control.
3. A method for the comparison of compounds which induce mRNA degradation, comprising separately contacting the compounds with a DNA expression system which in the absence of the compounds is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, measuring the detectable signal in the presence of each test compound and comparing the signals obtained.
4. A reporter gene DNA expression system comprising a gene coding for expression of a protein having a detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences

comprising appropriate expression control elements and DNA corresponding to at least one copy of a mRNA instability sequence.

5. A stably transfected cell line comprising a reporter gene DNA expression system according to claim 4.
6. An assay system for the identification of compounds which destabilise mRNA comprising  
a reporter gene DNA expression system as defined in claim 4, and  
a control DNA expression system which comprises a gene coding for expression of the protein having the detectable signal, wherein the gene comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements but lacking any functional mRNA instability sequence.
7. An assay system comprising  
a stably transfected cell line according to claim 5, and  
a stably transfected cell line comprising a control DNA expression system as defined in claim 6.
8. A stably transfected cell line comprising a reporter gene DNA expression system according to claim 4 and a control gene DNA expression system, said control gene DNA expression system comprising a gene coding for expression of a protein having a detectable signal which is different than the protein of the reporter gene DNA expression system and wherein said control gene DNA expression system comprises DNA coding for the amino acid sequence of the protein together with associated 5' and 3' UTR sequences comprising appropriate expression control elements but lacking any functional mRNA instability sequence.
9. An assay system comprising a stably transfected cell line according to claim 8.

10. A compound which destabilises mRNA when identified by a method according to any one of claims 1-3, or by use of a DNA expression system according to claim 4, a cell line according to claim 5 or 8, or an assay system according to claim 6, 7 or 9.
11. Use of a compound according to claim 10 for the prophylaxis or treatment of a disease or medical condition which involves inappropriate mRNA stabilisation and/or accumulation and undesirable protein expression.

**ASSAY FOR IDENTIFYING COMPOUNDS WHICH**  
**AFFECT STABILITY OF mRNA**

**ABSTRACT**

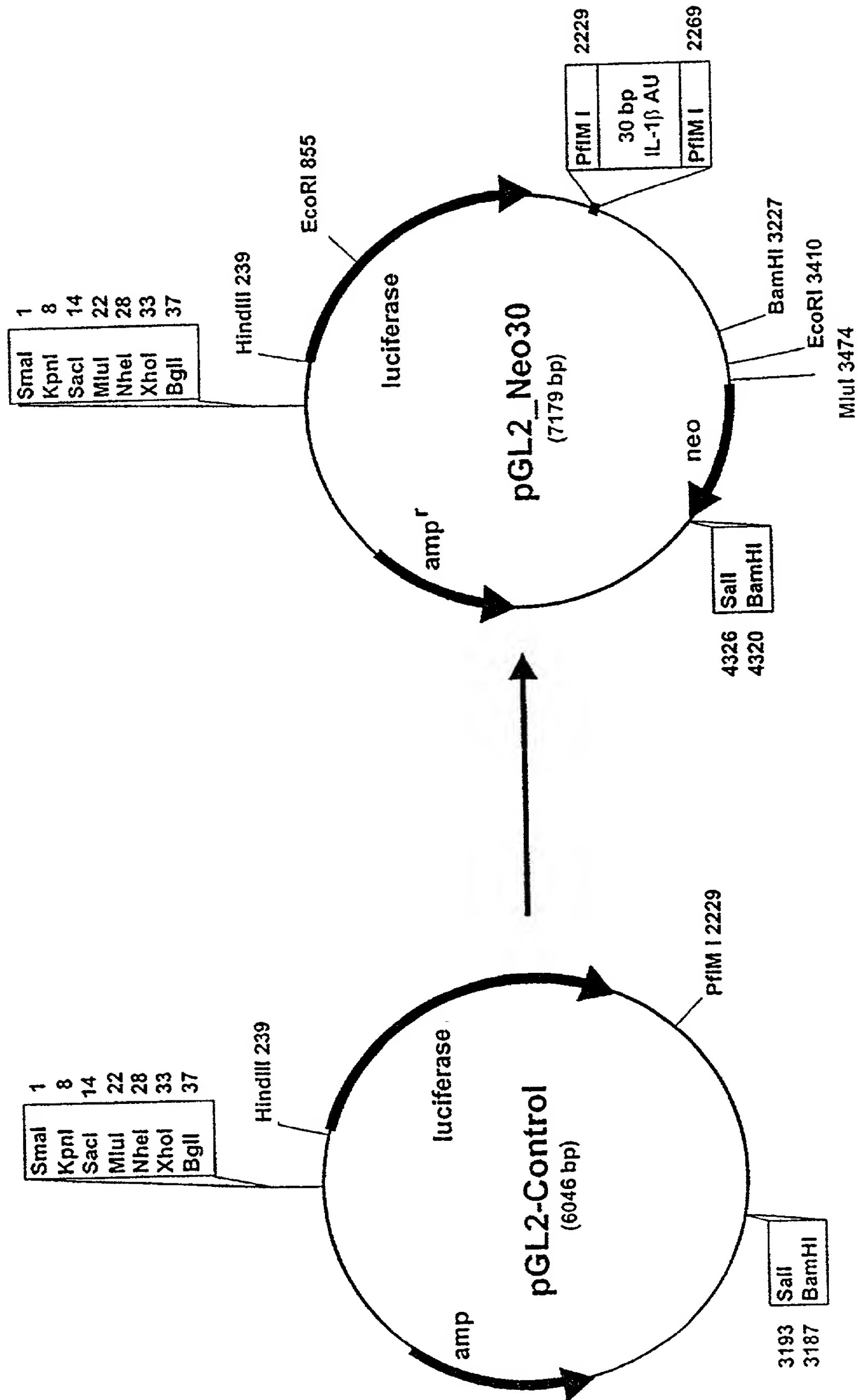
A method is provided for the identification of a compound which affects mRNA stability, in particular induces mRNA degradation, in which a DNA expression system which in the absence of test compound is capable of expressing a protein having a detectable signal, wherein the mRNA which codes for the protein and which is transcribed from the expression system comprises at least one copy of a mRNA instability sequence, is contacted with a test compound and the detectable signal is measured in the presence of the test compound and compared with a control. The method may be used to identify compounds which induce degradation of mRNA, e.g. cytokine (e.g. IL-1 $\beta$ ) mRNA, which when inappropriately stabilised can give rise to diseases or medical conditions, e.g. cytokine induced inflammatory disease.

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\* GGACCAAAGG CGGCCAGGAT ATAAGTACT TCACCATGCA  
 ATTTGTGTCT TCCTAAAGAG AGCTGTACCC AGAGAGTCCT  
 GTGCTGAATG TGGACTCAAT CCCTAGGGCT GGCAGAAAGG  
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FIGURE 1





**FIGURE 3**

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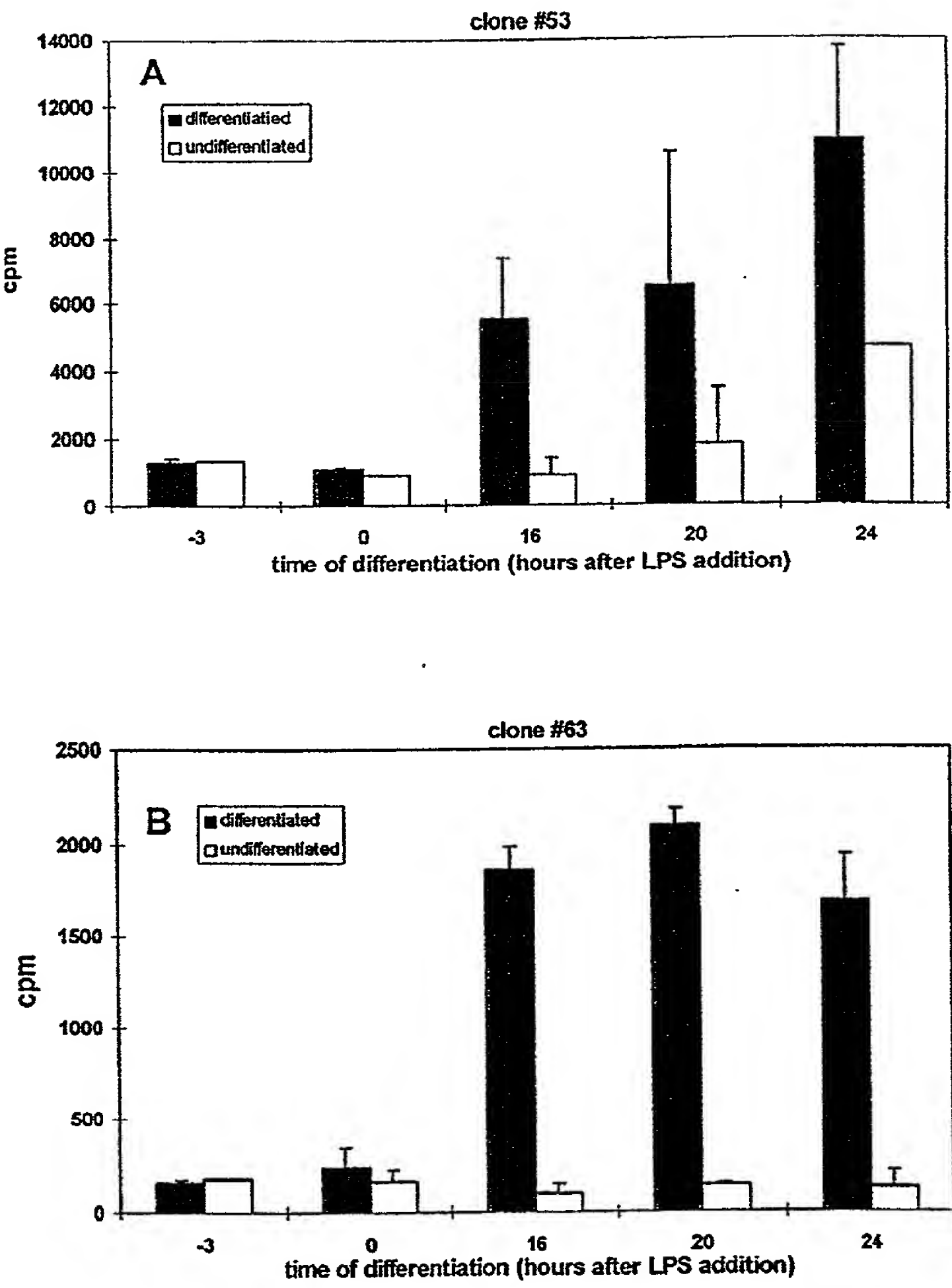


FIGURE 4



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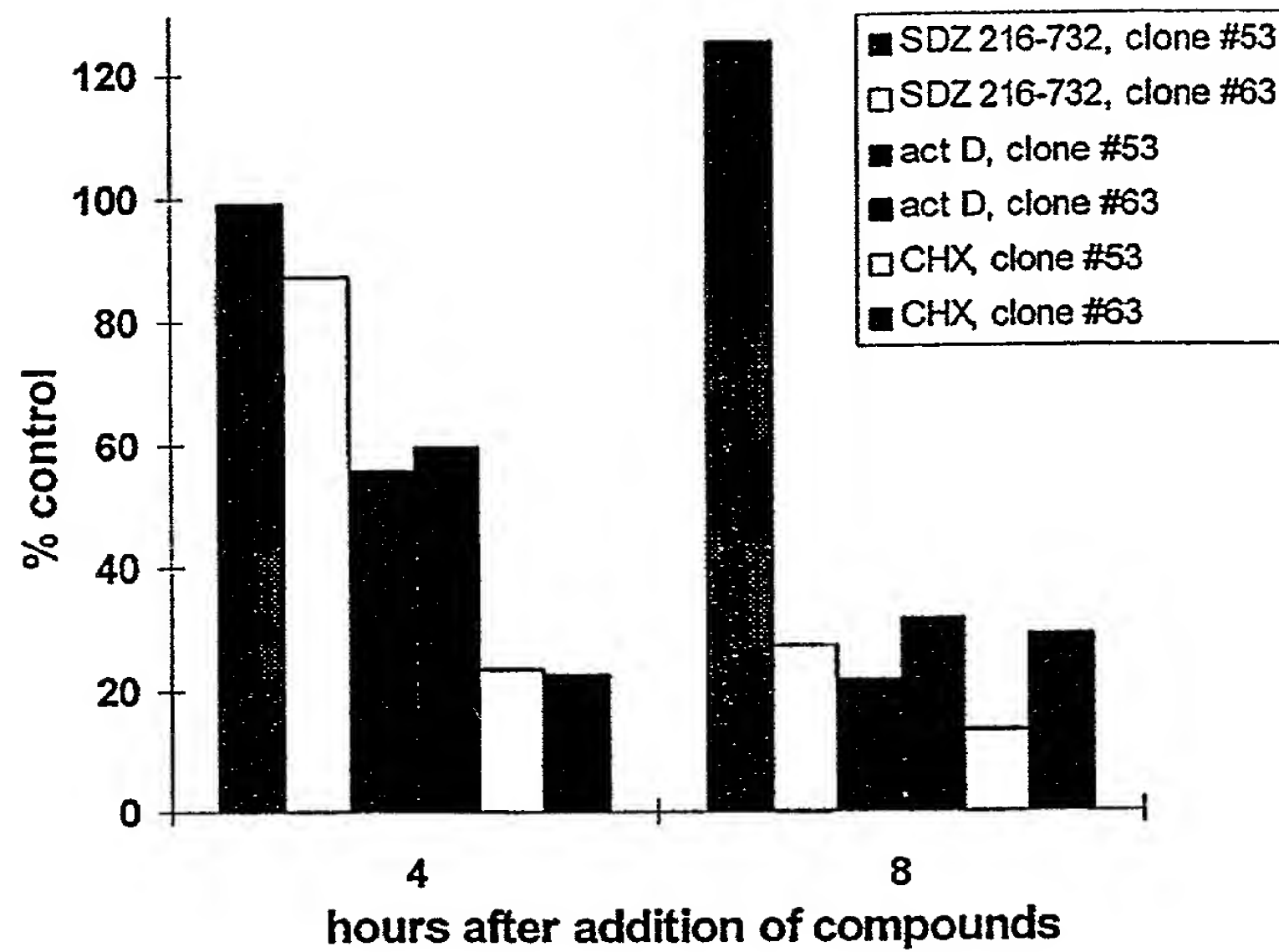


FIGURE 5

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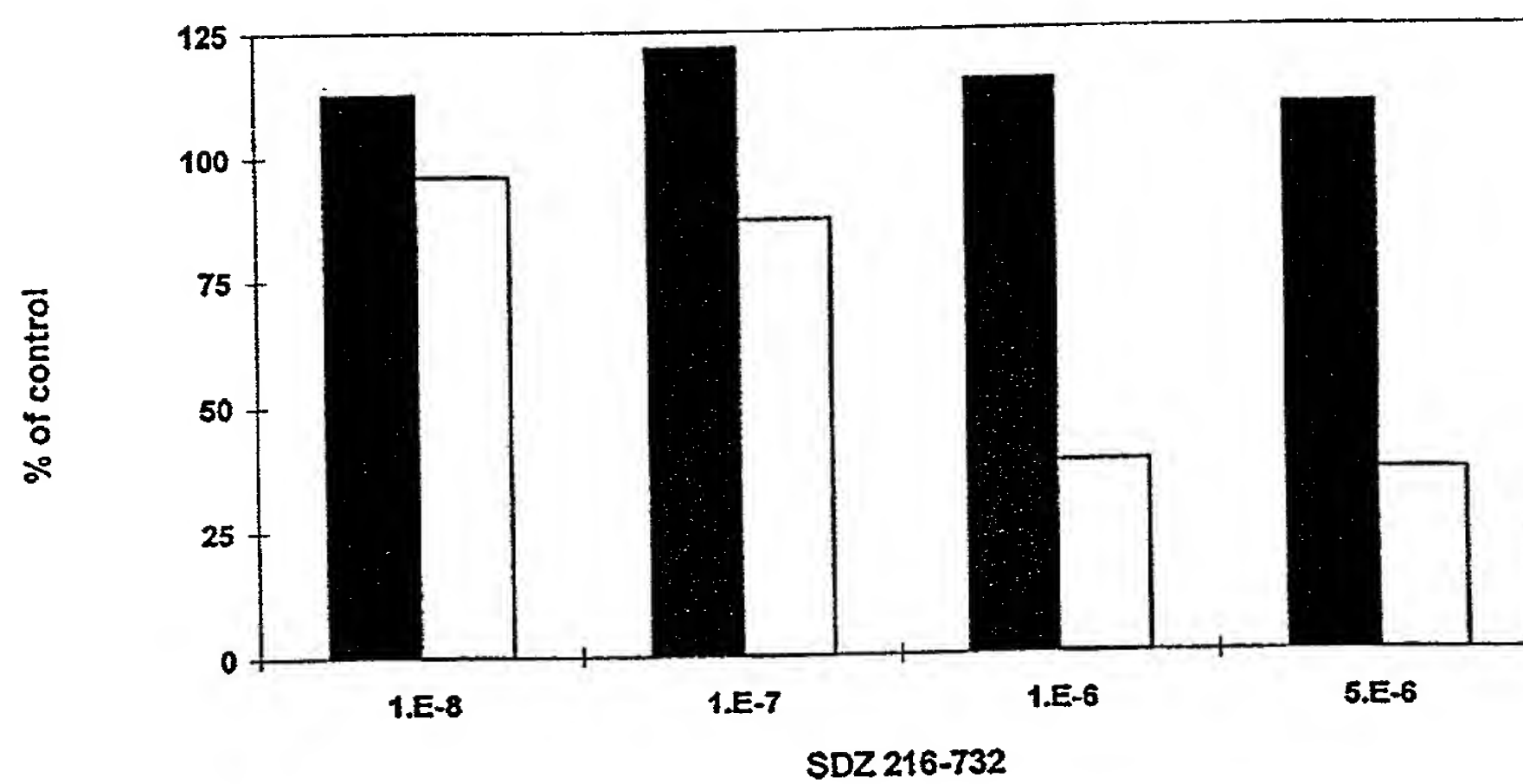
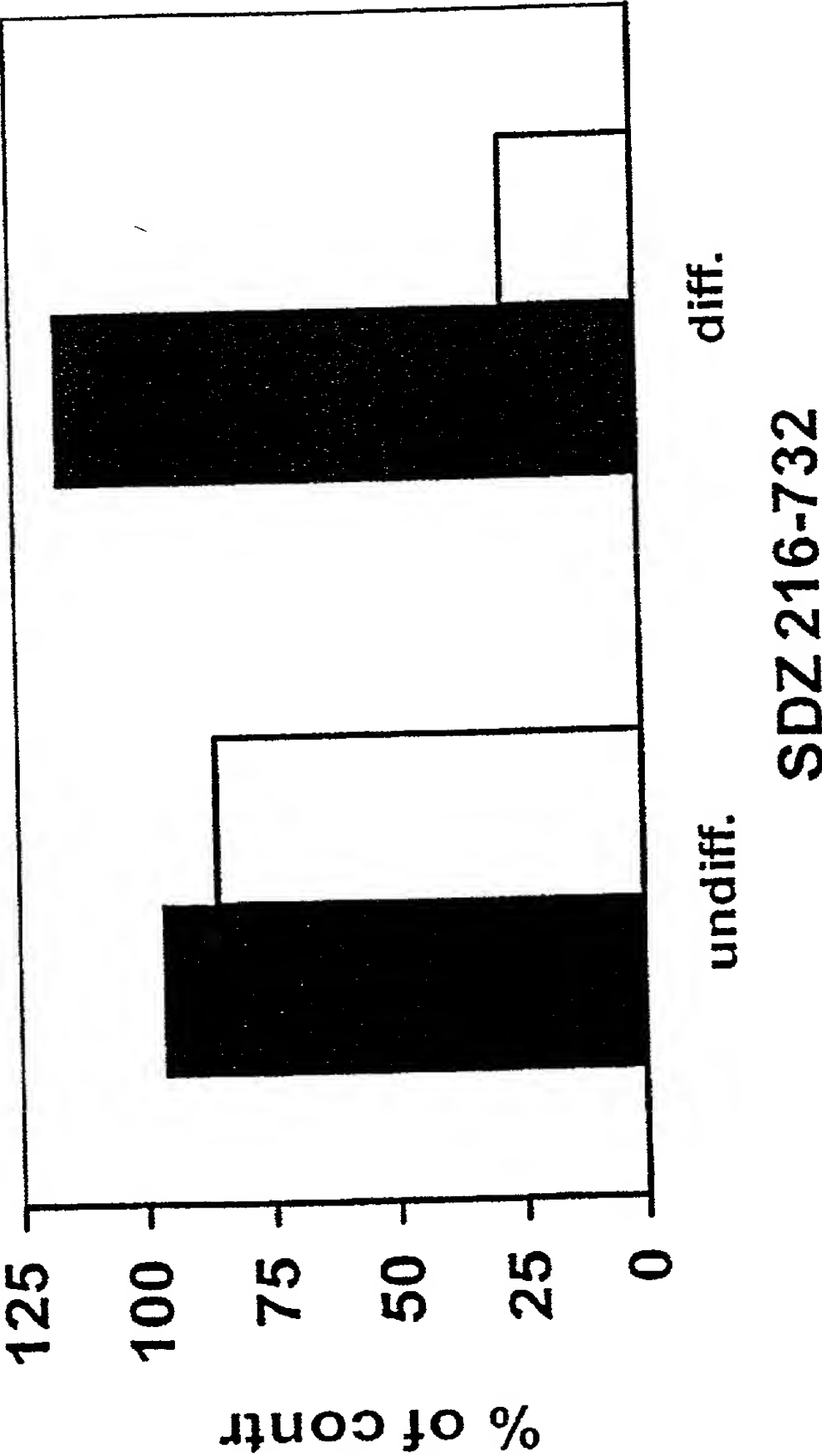
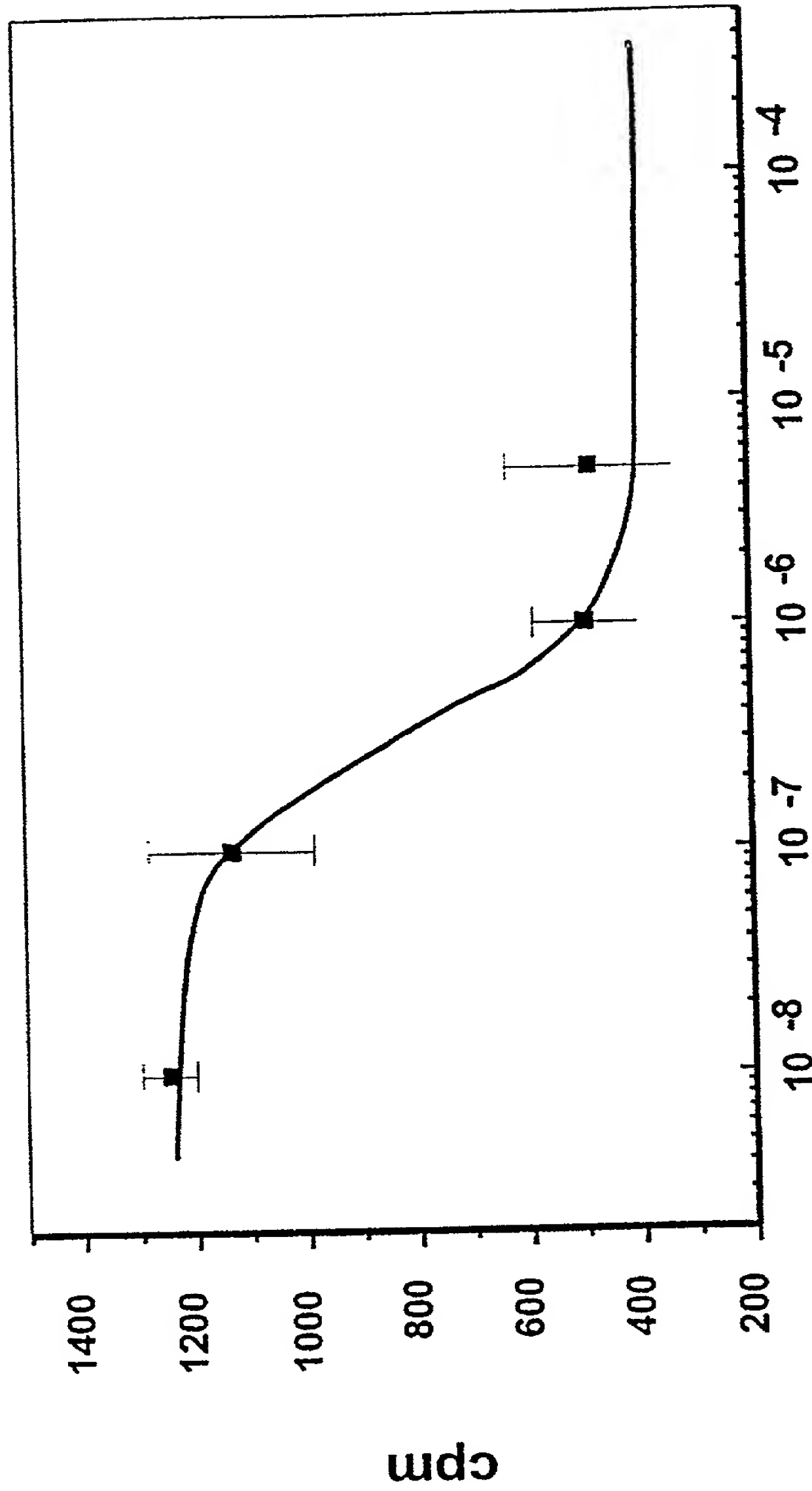


FIGURE 6



SDZ 216-732

FIGURE 7



SDZ 216-732

FIGURE 8

## Declaration for Patent Application

Docket Number: 1556.0290000/RWE

As a below named inventor, I hereby declare that:

My residence, mailing address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled:

Assay for Identifying Compounds Which Affect Stability of mRNA,

the specification of which is attached hereto unless the following box is checked:

- ☒ was filed on December 23, 1999;  
as United States Application Number or PCT International Application Number PCT/CA99/01235; and  
was amended on \_\_\_\_\_ (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56, including for continuation-in-part applications, material information that became available between the filing date of the prior application and the national or PCT international filing date of the continuation-in-part application.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or (f), or § 365(b) of any foreign application(s) for patent, inventor's or plant breeder's rights certificate(s), or § 365(a) of any PCT international application, which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent, inventor's or plant breeder's rights certificate(s), or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Claimed

9828709.7  
(Application No.)

Great Britain  
(Country)

24 December 1998  
(Day/Month/Year Filed)

☒ Yes ☐ No

\_\_\_\_\_  
(Application No.)

\_\_\_\_\_  
(Country)

\_\_\_\_\_  
(Day/Month/Year Filed)

☐ Yes ☐ No

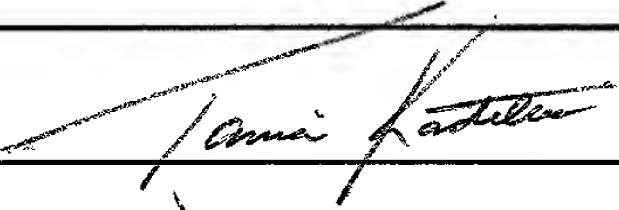
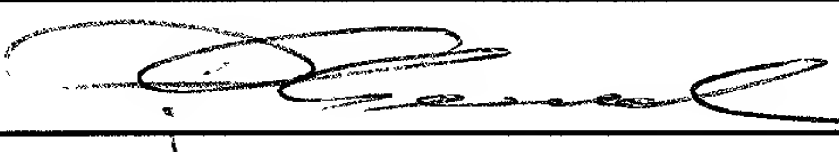
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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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